Nonrandom Patterns of Genetic Admixture Expose the Complex Historical Hybrid Origin of Unisexual Leaf Beetle Species in the Genus *Calligrapha*

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Submitted March 10, 2014; Accepted July 19, 2014; Electronically published December 2, 2014

Online enhancement: appendix. *Dryad data:* http://dx.doi.org/10.5061/dryad.b8h1n.

ABSTRACT: Many unisexual animal lineages supposedly arose from hybridization. However, support for their putative hybrid origins mostly comes from indirect methodologies, which are rarely confirmatory. Here we provide compelling data indicating that tetraploid unisexual *Calligrapha* are true genetic mosaics obtained via analysis of mitochondrial DNA (mtDNA) and allelic variation and coalescence times for three single-copy nuclear genes (*CPS*, *HARS*, and *Wg*) in five of six unisexual *Calligrapha* and a representative sample of bisexual species. Nuclear allelic diversity in unisexuals consistently segregates in the gene pools of at least two but up to three divergent bisexual species, interpreted as putative parentals of interspecific hybridization crosses. Interestingly, their mtDNA diversity derives from an additional yet undiscovered older evolutionary lineage that is possibly the same for all independently originated unisexual species. One possibly extinct species transferred its mtDNA to several evolutionary lineages in a wave of hybridization events during the Pliocene, whereby descendant species retained a polymorphic mtDNA constitution. Recent hybridizations, in the Pleistocene and always involving females with the old introgressed mtDNA, seemingly occurred in the lineages leading to unisexual species, decoupling mtDNA introgression (and inferences derived from these data, such as timing and parentage) from subsequent acquisition of the new reproductive mode. These results illuminate an unexpected complexity in possible routes to animal unisexuality, with implications for the interpretation of ancient unisexuality. If the origin of unisexuality requires a mechanism where (1) hybridization is a necessary but insufficient condition and (2) multiple bouts of hybridization involving more than two divergent lineages are required, then the origins of several classical unisexual systems may have to be reassessed.

Keywords: ancient asexual, hybridization, introgression, parthenogenesis, scnDNA, speciation.

Introduction

Unisexuality refers to a state in which a species or population is composed of individuals of a single (female) sex, while unisexual reproduction is the process whereby eggs develop without fertilization (Simon et al. 2003). This condition is a rare reproductive strategy among animals. However, it poses well-known fundamental conceptual challenges as to why it is not the prevalent strategy (the so-called paradox of sex; Williams 1975; Maynard Smith 1978) or, conversely, why it occurs at all and is maintained (e.g., asexual scandals; Judson and Normark 1996). Nonetheless, considering its scatter over the predominantly sexual metazoan tree of life, unisexuality also poses more case-specific and difficult-to-answer evolutionary questions regarding the origin of each unisexual phylogenetic branch.

Depending on the system investigated, the origins of unisexual species have been explained by different mechanisms. There are relatively simple explanations for the rise of unisexuality in some cases (Simon et al. 2003), based on the mutation and/or inactivation of genes important for meiosis (spontaneous origins) and the transmission of these genetic factors via gene flow in species polymorphic for this condition (contagious origins). Alternatively, unisexuality can be attained through reproductive manipulation of the host by endosymbiotic bacteria, such as *Wolbachia*, *Rickettsia*, or *Spiroplasma* (Werren et al. 2008; Engelstädter and Hurst 2009; infectious origins per Simon et al. 2003). These modes of origin—specifically, spontaneous and contagious origins—typically give rise to lineages that show mixed reproductive modes. Yet the vast majority of species in evolutionary lineages that develop obligate unisexuality seem to derive from a different mechanism altogether: interspecific hybridization (Bullini 1994; Lodé 2013).

The role of hybridization and its potential to generate diversity has been typically neglected in evolutionary studies of animals, yet there are estimates of up to 10% of animals being able to hybridize (Mallet 2007), and hybridization has been invoked to explain the origin of most

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Am. Nat. 2015. Vol. 185, pp. 113–134.
DOI: 10.1086/678408

unisexual animals (Simon et al. 2003). Indeed, all unisexual vertebrates seem to have a hybrid origin (Avise et al. 1992; although there is some controversy on this issue: Sinclair et al. 2010). However, the majority of metazoan unisexuals are spread across other phyla, and demonstration of their putative evolutionarily hybrid origins has used a range of mostly indirect methods, which are rarely confirmatory.

The earliest indication of interspecific hybridization leading to the derivation of unisexual lineages may be the recognition of intermediate phenotypes between sexually reproducing species in populations with highly biased sex ratios or shown to reproduce in the absence of mating (e.g., Hubbs and Hubbs 1932). However, indisputable empirical demonstration of this evolutionary route comes from inducing the hypothesized interspecific crosses in the laboratory and recovering the expected unisexual phenotypes (i.e., repeating evolution under controlled conditions). Laboratory synthesis of unisexuals from bisexual parentals has been successful, for instance, among the vertebrates in *Poeciliopsis* and *Cobitis* fishes (Schultz 1969; Choleva et al. 2012), *Pelophylax* frogs (Hotz et al. 1985), and *Aspidoscelis* lizards (Lutes et al. 2011) and among the insects in *Warramaba* grasshoppers (White et al. 1977) and *Muellerianella* planthoppers (Drosopoulos 1978). This approach still entails many practical difficulties. Most critically, it is only possible for unisexual species or populations that are recursively produced in nature or that originated recently so that all the species involved are extant, and in principle, it is not feasible to test historical hybrid origins of unisexual evolutionary lineages. Thus, the hybrid origin of most unisexual species or lineages subsequently acquiring this reproductive mode is inferred from indirect evidence, and the bulk of data comes from the study of genetic characters in the fields of cytogenetics (via the analysis of ploidy), genetics, and phylogenetics. In the latter two particularly, the underlying rationale is that, by virtue of their anastomosed origin, the genetic makeup of unisexual hybrid species will be more diverse than that of their bisexual counterparts, thus increasing their heterozygosity or generating species polyphyly, respectively.

The earliest attempts at showing higher heterozygosities in putatively hybrid unisexuals exploited the comparison of allozyme profiles. Many classical studies showed the expected pattern in almost all putative hybrid unisexual systems known at the time, and they were also, via comparison with allozyme profiles from bisexual species, able to contrast putative parental hypotheses (e.g., Parker and Selander 1976; Vrijenhoek et al. 1977; Honeycutt and Wilkinson 1989; Moritz et al. 1989; Johnson 1992; Tomiuk and Loeschcke 1992; Bogart and Klemens 1997; Taylor and Ó Foighil 2000). These methods typically detect only a subset of genetic variation, and homology assignment is troublesome, so they were soon superseded by DNA-based techniques that allowed phylogenetic tests of the origins of unisexuality. When a particular unisexual taxon is found to be polyphyletic for a single DNA marker, usually mitochondrial DNA (mtDNA) data, a hybrid origin is considered to be a more plausible explanation than alternative hypotheses. This reasoning was used to favor the hypothesis of hybridization, for example, in unisexual *Lasaea* clams (Ó Foighil and Smith 1995) and *Daphnia* water fleas (Dufresne and Hebert 1994). However, while this type of mtDNA polymorphism could certainly be explained by some kind of hybridization-mediated introgression, this phylogenetic pattern may be retrieved under only certain conditions, such as multiple recent origins of unisexuality from a polymorphic bisexual stock (thus with introgression not necessarily related to unisexuality) or multiple recent origins with symmetric contribution of mtDNA from putative parental species. Hybridization, bringing together genomic elements resulting from different evolutionary histories, is expected to result in incongruent phylogenetic signals from independent genetic markers (Brower et al. 1996). Thus, several studies trying to unravel the hybrid origins of unisexuality have investigated this type of incongruence between mtDNA and nuclear data (e.g., Murphy et al. 2000; Morgan-Richards and Trewick 2005; Gómez-Zurita et al. 2006). Nevertheless, phylogenetic incongruence may be caused by processes other than hybridization (Brower et al. 1996; Funk and Omland 2003; Toews and Brelsford 2012), and this approach is rarely conclusive relative to hybrid origins. Analysis of allelic variation in single-copy nuclear genes (scnDNA) is more practical and essentially undertaken with the same idea underlying the comparison of allozyme profiles. This approach has been used to provide relative timing for the origin of unisexual species (Birky 1996; Welch and Meselson 2000; Schwander et al. 2011), but the characterization of allelic variation in scnDNA from individuals of species with alternative reproductive modes can be used to confirm the hybrid origins of unisexual lineages as well, including historical events intractable in the laboratory. If a unisexual species were hybrid in origin, it would inherit alleles from each bisexual parent. Phylogenetic inference based on allele data should thus segregate unisexual alleles in divergent clades that would be related to their respective parental species, an unequivocal signature of hybridization (unless some process of genomic purge takes place; e.g., Dufresne and Hebert 1994). However, even if these patterns are retrieved, causality between hybridization and the actual origin of unisexuality is difficult to infer. For example, spontaneous mutations inducing a unisexual phenotype on an admixed sexual population (e.g., Schwander and Crespi 2009) or the rare fertilization of unisexual eggs by sperm from a different sexual species

(e.g., Neiman et al. 2011) could generate the same pattern as expected under hybrid origins of unisexuality (and, in the latter case, polyploidization intimately associated with these traits).

Few studies have used scnDNA variation to investigate the origins of unisexual lineages (aphids: Delmotte et al. 2003; stick insects: Buckley et al. 2008; nematodes: Lunt 2008). Here we use this approach to test the hypothesis of hybrid origins of unisexual species or their ancestors in the leaf beetle genus *Calligrapha*, which was originally formulated based on incongruent phylogenetic patterns between mtDNA and nuclear phylogenies (Gómez-Zurita et al. 2006). *Calligrapha* is a species-rich New World beetle genus with nearly 40 species distributed in North America, including up to six known obligate unisexual taxa and one reported as facultative (Brown 1945; Robertson 1966; Gómez-Zurita et al. 2004). Unisexuality is an extremely rare reproductive mode in the beetle family Chrysomelidae, and when it occurs, it is typically associated with peripheral populations of otherwise sexually reproducing species (Cox 1996). Yet in *Calligrapha*, the transition to unisexuality occurred many times independently, which makes it a very interesting evolutionary oddity, covered in the classical treatises on the evolution of sex (e.g., Maynard Smith 1978; Bell 1982). Unisexuality in *Calligrapha* has been unequivocally associated with tetraploidy (Robertson 1966), which likely arose via allotetraploidy (Gómez-Zurita et al. 2006). This, together with phylogenetic reasoning, suggests their hybrid origin. We use this circumstance to our advantage to formulate our current working hypothesis: If unisexuality arose in tetraploid *Calligrapha* species owing to hybridization between diploid bisexual species, then by resolving alleles of scnDNA genes copies using a polymerase chain reaction (PCR)/cloning strategy, we expect to find a minimum of two divergent allelic variants in unisexual species. Moreover, the phylogenetic position of these alleles relative to the homologue copies from bisexual species should reveal the parental evolutionary lineages involved in these hybridization events.

Material and Methods

Taxon Sampling

For this study, we investigated a total of 50 individual *Calligrapha* specimens in 19 North American species, including five of the six thelytokous, obligate unisexual species (table 1). Bisexual species were represented by one external outgroup considered for phylogenetic tests (*C. dislocata*; see below) and 13 of 18 species belonging to the clade where unisexual taxa originated, including all potential parental evolutionary lineages (Gómez-Zurita et al. 2006).

Laboratory Methods

Beetle total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen Iberia, Madrid), following manufacturer protocols. Three single-copy nuclear protein-coding genes were selected to investigate individual heterozygosity and scnDNA gene/allele genealogies: the carbamoyl phosphate synthase (*CPS*) domain of the *CAD* locus (*rudimentary* in *Drosophila*), histidyl-transfer RNA (tRNA) synthetase (*HARS*, also known as *Aats-his* in *Drosophila*), and wingless (*Wg*). The fragment of the *CPS* domain was PCR amplified for most samples using primers CD439F (5'-TTC AGT GTA CAR TTY CAY CCH GAR CAY AC-3') and CD668R (5'-ACG ACT TCA TAY TCN ACY TCY TTC CA-3') or CD688R (5'-TGT ATA CCT AGA GGA TCD ACR TTY TCC ATR TTR CA-3'; Wild and Maddison 2008), but all alignments were cut to the region corresponding to the shorter fragment (obtained with CD668R). *HARS* was amplified using degenerate primers designed for our study: HARSf (5'-TAY GAY YTG AAR GAY CAR GG-3') and HARSr (5'-YTC YTC RCA RTG YTG CAR YTG-3'). Finally, *Wg* was amplified with primers Wg550F (5'-ATG CGT CAG GAR TGY AAR TGY CAY GGY ATG TC-3') and WgAbRZ (5'-CAC TTN ACY TCR CAR CAC CAR TG-3'), which preferentially target one of the gene paralogues of the *Wnt* family, which are recognizable by size (Wild and Maddison 2008). Polymerase chain reactions used 3 mM $MgCl_2$, 0.2 μM of each *CPS* or *Wg* primer, or 0.4 μM of each *HARS* primer, and both conventional BIOTAQ DNA Polymerase and high-fidelity BIO-X-ACT Long DNA Polymerase (both from Bioline, London) were tested. Since there were no apparent differences in performance, sequence quality, and, critically, in the proportion of recovered PCR artifacts (see below), the conventional polymerase was used to produce the final data set. Cycling conditions typically used a touchdown protocol of 25 cycles with annealing temperature decreasing from 65° to 40°C (45 s) and 10 cycles at 40°C (45 s), denaturation at 95°C for 3 min in the first cycle and 30 s in the remaining cycles, and elongation at 72°C for 60 s in all cycles, except in the last cycle, in which it lasted 10 min. Some *CPS* and *Wg* fragments were amplified using alternative touchdown protocols with annealing temperatures decreasing from 65° to 50°C or from 60° to 45°C, respectively. The PCR products were separated in 1.5% agarose gels to check success and quality of amplification. In the few cases where no amplified product was visible on gel for the *HARS* and *Wg* genes, 1 μL of the original PCR reaction was used as a template for reamplification under the same conditions as above. When this happened for *CPS* (about half of the reactions), an amplicon obtained using the primers CD439F and CD851R (5'-GGA TCG AAG CCA TTH ACA TTY TCR TCH ACC AT-3'; Wild and Maddison 2008) served as a template for a heminested reamplification

Table 1: Taxon sampling in *Calligrapha*, including host plant, specimen voucher number, source, and molecular data

Species	Host plant	Voucher no.	Source	Molecular data[a]
Unisexual species:				
C. alnicola Brown	*Alnus*			
		IBE-JGZ-C275	Canada: QC, Cowansville	C (18/15/10)
		IBE-JGZ-0250	Canada: QC, Quyon	C (13/12/19)
		IBE-JGZ-0563	USA: MN, Cook County	C (10/9/18)
		IBE-JGZ-C169	USA: WI, Vilas County	C (12/18/18)
C. apicalis Notman	*Alnus*			
		IBE-JGZ-0552	Canada: QC, Cowansville	C (23/12/18)
		IBE-JGZ-C276	Canada: QC, Cowansville	C (9/14/20)
C. suturella Schaeffer	*Salix*			
		IBE-JGZ-0331	Canada: MB, Ameer	C (24/16/14)
		IBE-JGZ-0335	Canada: MB, Ameer	C (18/13/13)
		IBE-JGZ-0129	Canada: QC, Stoneham	C (24/18/13)
		IBE-JGZ-C103	USA: ME, Cumberland County	C (15/18/15)
		IBE-JGZ-C104	USA: ME, Cumberland County	C (11/14/16)
		IBE-JGZ-0333	USA: ME, Kennebec County	C (13/18/14)
		IBE-JGZ-0555	USA: ME, Kennebec County	C (16/7/12)
		IBE-JGZ-0130	USA: ME, Somerset County	C (11/14/10)
		IBE-JGZ-0334	USA: MI, Grand Traverse County	C (12/14/15)
		IBE-JGZ-C102	USA: WI	C (15/16/21)
C. vicina Schaeffer[b]	*Cornus*			
		IBE-JGZ-C376	Canada: ON, Chesterville	C (15/18/19)
C. virginea Brown[b]	*Tilia*			
		IBE-JGZ-C135	USA: WI, Brown County	C (10/16/16)
		IBE-JGZ-C195	USA: WI, Brown County	C (16/22/15)
Bisexual species:				
C. alni Schaeffer	*Alnus*			
		IBE-JGZ-0126	Canada: QC, Chicoutimi	C (1/18/13)
		IBE-JGZ-C128	USA: MN, Hubbard County	C (15/21/19)
		IBE-JGZ-0116	USA: WV, Tucker County	C (13/9/19)
C. amator Brown	*Tilia*			
		IBE-JGZ-0247	Canada: ON, Selkirk	C (16/17/16)
C. confluens Schaeffer	*Alnus*			
		IBE-JGZ-0121	Canada: QC, East Angus	C (16/14/10)
		IBE-JGZ-0123	USA: VT, Caledonia County	C (18/18/16)
C. dislocata (Rogers)[c]	Asteraceae			
		IBE-JGZ-0394	USA: NM, Torrance County	D
C. floridana Schaeffer[b]	*Cornus*			
		IBE-JGZ-C318	USA: FL, Alachua County	D
C. ignota Brown	*Betula*			
		IBE-JGZ-0556	USA: MN, Anoka County	C (13/19/17)
		IBE-JGZ-C164	USA: WI, Door County	C (21/15/12)
C. multipunctata (Say)	*Salix*			
		IBE-JGZ-0336	Canada: MB, Strathclair	C (16/8/18)
		IBE-JGZ-CM064	Canada: QC, Saints-Anges	C (15/16/19)
		IBE-JGZ-C031	USA: ID, Bonner County	C (16/15/13)
		IBE-JGZ-C027	USA: OR, Baker County	C (13/17/14)
		IBE-JGZ-C041	USA: OR, Clatsop County	C (14/19/14)
C. philadelphica (L.)	*Cornus*			
		IBE-JGZ-C072	Canada: AB, Edmonton	D
		IBE-JGZ-C530	Canada: NB, Saint-Jacques	D
		IBE-JGZ-0283	Canada: ON, Ottawa	C (13/16/16)
		IBE-JGZ-C100	Canada: ON, Ottawa	C (19/12/16)
		IBE-JGZ-C091	Canada: ON, Jordan	C (19/16/16)
		IBE-JGZ-C500	USA: ME, Aroostook County	D
		IBE-JGZ-C182	USA: MN, Clay County	C (16/14/13)

Table 1 (*Continued*)

Species	Host plant	Voucher no.	Source	Molecular data[a]
C. pruni Brown[b]	*Prunus*			
		IBE-JGZ-C140	USA: IA, Sioux County	D
		IBE-JGZ-C133	USA: MN, Freeborn County	D
C. rhoda Knab	*Corylus*			
		IBE-JGZ-C163	USA: MN, Beltrami County	D
C. rowena Knab	*Cornus*			
		IBE-JGZ-C411	USA: NH, Hillsborough County	D
		IBE-JGZ-C547	USA: NH, Hillsborough County	D
C. scalaris (LeConte)	*Ulmus*			
		IBE-JGZ-0405	USA: ND, Pembina County	C (18/14/16)
		IBE-JGZ-C326	USA: OK, Roger Mills County	C (20/14/18)
C. spiraea (Say)	*Physocarpus*			
		IBE-JGZ-0114	USA: WV, Greenbrier County	D
C. verrucosa (Suffrian)[b]				
	Salix	IBE-JGZ-C066	USA: MT, Ravalli County	D

[a] C = cloning/sequencing of polymerase chain reaction products; D = direct sequencing of PCR products. Values in parentheses are the numbers of clones sequenced for carbamoyl phosphate synthase, histidyl-tRNA synthetase, and wingless, respectively.

[b] Taxa not used in Gómez-Zurita et al. (2006).

[c] Used only as outgroup for the analysis of time for the most recent common ancestors ranges (see "Material and Methods").

using either CD668R or CD688R. Finally, a few cases produced multiple bands, and the PCR products of the expected size were excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen Iberia, Madrid). The PCR products obtained from all individuals in the unisexual species and from two-thirds of individuals in the bisexual species (table 1) were cloned using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA), following manufacturer instructions. An average of 16 transformed colonies (ranging from 1 to 24) were selected for sequencing using BigDye 3.1 technology (Applied Biosystems, Foster City, CA) and universal M13(-21) and M13Rev primers. These same PCR products were also directly sequenced prior to cloning. The homologue sequences for several bisexual species were obtained only by direct sequencing (table 1).

Additionally, three mtDNA genetic markers (partial sequences of the cytochrome *c* oxidase subunits 1 and 2, *cox1* and *cox2*, and the large ribosomal RNA subunit, *rrnL*) were amplified following the same reaction mix described above and the same cycling conditions as in Gómez-Zurita et al. (2006). Sequences used in this study have been deposited in the European Nucleotide Archive (EMBL-EBI, Hinxton, UK) under accession numbers LK391972-LK392065 (*CPS* locus of *CAD*), LK392066-LK392155 (*HARS*), LK392156-LK392243 (*Wg*), LK392244-LK392265 (*rrnL*), and LK392266-LK392295 (*cox1-trnL-cox2*).

Sequence Edition and Refinement

Sequences were edited using Geneious Pro 5.3.6 (Biomatters, Auckland). A few sequences had low-quality ends (43 positions from the 5'-end and 40 positions from the 3'-end of the *HARS* gene and 30 positions from the 5'-end of *Wg*), and all others were trimmed to these positions to avoid the impact of missing data in subsequent analyses. Heterozygote double peaks in directly sequenced PCR products were coded using International Union of Pure and Applied Chemistry ambiguity codes, and the software PHASE 2.1 (Stephens et al. 2001; Stephens and Scheet 2005) was subsequently used (via SeqPHASE; Flot 2010) to infer their most likely state based on the comparison of samples providing unambiguous sequences.

Clones (regardless of their being obtained using high-fidelity or conventional *Taq* polymerases) exhibited high levels of intraindividual polymorphism incompatible with the species ploidy levels and with the sequence directly obtained from the same PCR products. These were interpreted as random PCR artifacts, in some cases due to early *Taq* polymerase in vitro mutations (Tindall and Kunkel 1988) but also because of the formation of chimeras due to priming by incomplete PCR products in samples polymorphic (heterozygous) for the locus of interest (Meyerhans et al. 1990; Bradley and Hillis 1997). Previous empirical work has shown that both sources of error are not negligible. For instance, nearly 40% of sequences obtained by PCR amplification of a known 1,000 bp sequence were shown to contain one or more artificial substitutions (Kobayashi et al. 1999), and Lahr and Katz (2009) found that under some extreme PCR conditions, up to 70% of the obtained products were chimeric artifacts. Our empirical corroboration of this problem required further refinement to obtain canonical data sets for analysis.

First, the direct sequence of each PCR product was compared with its respective set of cloned sequences. Point mutations present in clones but absent (without any evidence for double peaks) from direct sequencing were interpreted as polymerase errors, as were a few observed mutations resulting in stop codons, and restored to the state observed in the direct sequence. This is one of two sound procedures used in the few studies that take into account polymerase errors to assess allelic variation in nonmodel organisms (e.g., Buckley et al. 2008; Schwander et al. 2011; Harpke et al. 2013). A more rigorous but less cost-effective—and, therefore, not so frequently used—alternative duplicates both PCR and cloning experiments to purge inconsistencies (e.g., Lukas and Vigilant 2005; Schwander et al. 2011). Subsequently, clones from the same PCR product were collapsed to different alleles and annotated with abundances. In some cases, more than two or four sequence variants were obtained for diploid and tetraploid species, respectively, and the observed variation was compatible with some of these variants being chimeras. Chimeras were objectively detected using the UCHIME algorithm (Edgar et al. 2011), as implemented in USEARCH 6.0.203 (Edgar 2010), a method based on the analysis of three-way sequence alignments of query and putative parental sequences and the identification of alternating sequential tracts of sequence similarity with alternative parental sequences. First, we used the so-called de novo mode for chimera detection, which targets alternating variability patterns in sequence tracts but also frequency information, assuming that parent sequences should be more abundant than their chimeras. All detected chimeras were removed from the data set. However, this assumption was violated in our data set in some cases, specifically when several haplotypes were recovered with the same frequency (e.g., singletons). When this happened, the remaining clones were subjected to an additional strategy, the reference mode (Edgar et al. 2011). This strategy relies on comparisons with potential parent sequences as provided by the user, which in our case were all direct sequences plus all the alleles represented by more than three clones in any of the sets. The trade-off between the specificity and sensitivity of the method was modulated for our low-diversity data by retuning the algorithm default parameters to minimum score threshold (-minh = 0.001), minimum divergence (-mindiv = 0.001), minimum number of differences in each segment (-mindiffs = 1), and weight of abstention vote (-xa = 30). Sequential objective removal of these artifacts rendered data sets with intraindividual diversity, in most cases, compatible with the species ploidy levels (i.e., up to two sequences for diploid bisexual species and four alleles at most for tetraploid unisexual species).

Estimation of Population Genetics Parameters and Intralocus Recombination

Once chimeric data were purged from each scnDNA data set, the respective length-invariable sequence data sets were manually aligned with the phased sequences from direct sequencing. Aligned sequence data for each marker were used to estimate genetic diversity parameters, including number of alleles, number of segregating sites, and nucleotide diversity for every locus and species using DnaSP 5.0 (Librado and Rozas 2009). Sequence alignments are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.b8h1n (Montelongo and Gómez-Zurita 2014). These data were compared with equivalent measures for one mtDNA marker, the 3'-end of the *cox1* gene (data newly generated and from Gómez-Zurita et al. 2006). Differences in measures of polymorphism were compared for each marker using the Mann-Whitney test in SPSS 20.0 (IBM, Armonk, NY). Data from each locus objectively free from chimeras were additionally tested for intralocus recombination using a range of available methods, including pairwise homoplasy index (PHI) tests (Bruen et al. 2006), as implemented in SplitsTree 4.12.8 (Huson and Bryant 2006), and RDP, GENECONV, MaxChi, Bootscan, and SiScan methods, all implemented in RDP4 4.19 (Martin et al. 2010, and references therein for each specific methodology).

Coalescent-Based Approaches to Intraspecific Genomic Mosaicism

The same data matrices were used individually for phylogenetic tests addressing the question of hybrid origins. Allele genealogies for each nuclear locus were inferred based on statistical parsimony, minimizing the effects of potential homoplasy due to recurrent mutations in the sample, by means of TCS 1.21 (Clement et al. 2000). The obtained genealogies showed the phylogenetic dispersion of intra- and interspecific genetic diversity in the sample and allowed mapping of reproductive strategies for a qualitative assessment of genetic mosaicism and recognition of phylogenetic affinities of unisexual diversity.

Allele genealogies show a snapshot, a sample of the evolution of the markers studied here, but in order to evaluate whether the obtained trees were predictive of the patterns expected under the hypothesis of interspecific genetic mosaicism, versus the essentially random effects of incomplete lineage sorting, we evaluated one hypothetical expectation for historical hybrid origins based on random samples of trees obtained under a neutral coalescent process. Coalescence times for alleles drawn from a sample of individuals in unisexual species are expected to be significantly higher than those for bisexual species and compatible with

interspecific divergence if their origin involved several species. Conversely, the times for the most recent common ancestors (TMRCA) of the different species would not show significant differences, irrespective of their reproductive mode (and ploidy), if the origin of their genetic diversity were not mosaic through interspecific hybridization. These analyses were run in BEAST 1.7.5 (Drummond et al. 2012) for each chimera-free gene data set individually, specifying the evolutionary model as estimated with MrModeltest 2.3 (Nylander 2004) based on the Akaike information criterion (AIC) and using a range of analytical conditions affecting evolutionary rate—strict (SC) and uncorrelated lognormal (ULN) clocks—and branching model—constant size (CSc), logistic growth (LGc), and Bayesian skyline (BSc) coalescents and Yule (Ysp) and birth-death (BDsp) speciation models. These data sets included the respective homologous sequence from a suitable outgroup (*C. dislocata*), and root age was modeled following a normal distribution with mean = 5 and standard deviation = 0.5, based on previously published data (Gómez-Zurita et al. 2006). Uniform wide priors were used for clock-related parameters, and everything else was set as defaults. Markov chain Monte Carlo searches ran for 15 million generations, and trees were sampled every 1,000 generations. Means and confidence intervals for the TMRCA of unisexual and bisexual species were computed from trees drawn after removing the nonstationary phase of the analysis in Tracer 1.5 (Rambaut and Drummond 2007).

Inference of mtDNA Phylogeny

Each mtDNA marker was aligned separately using the G-INS-i algorithm in MAFFT 7 (Katoh and Standley 2013). Data were concatenated and partitioned using the best scheme and substitution model as selected by PartitionFinder 1.0.1 (Lanfear et al. 2012), based on the AIC, through exhaustive searches among all possible partition schemes comparing the models implemented in MrBayes and considering a single set of branch lengths across partitions. MrBayes 3.2.1 (Ronquist et al. 2012) was used for tree inference on the CIPRES Science Gateway 3.3 (Miller et al. 2010). Tree search consisted of two independent runs of three hot chains and one cold chain for 100 million generations, sampling every 2,000 generations. Parameters were estimated for each unlinked partition, allowing partitions to evolve under different rates. The initial 25% of trees (12,500 trees) were discarded (burn-in) based on standard deviations of split frequencies below 0.01 and stability of log likelihoods.

Results

Intralocus Genetic Diversity

We cloned 38 specimens in 12 *Calligrapha* species, including all specimens available (19) for unisexual species (table 1). In total, we obtained 577 *CPS*, 576 *HARS*, and 591 *Wg* cloned sequences. Comparisons of these cloned sequences with their respective direct sequencing of the same PCR product allowed recognizing and editing 0.14%–0.17% point mutations that we interpreted as polymerase errors. Analysis of these corrected data applying the de novo strategy for identification of chimeric artifacts purged 45, 22, and 26 sequences from each marker, respectively, and a refined search using the so-called reference mode allowed identifying 32, 12, and 8 additional chimeras. Most of these chimeras were associated with sequences obtained from individuals in unisexual species (i.e., 83.1% in *CPS*, 100% in *HARS*, and 82.4% in *Wg*). The resulting purged data matrices were used to collapse the alleles represented in each individual, and the result was compatible with the known ploidy level for each species, that is, four alleles at most (most typically, three alleles) for tetraploid unisexual species (table 2) and two alleles at most for diploid bisexual species (table 3). The only exceptions were found for specimen IBE-JGZ-0126 of the diploid bisexual *C. alni*, where three different *Wg* sequences were retained, and for IBE-JGZ-C102 of the tetraploid unisexual *C. suturella*, where five *CPS* sequences were objectively filtered out of seven variants. However, one and two sequences, respectively, of these two sets were recovered from single clones and most likely represent unidentified chimeras (alleles 2_{Wg} and $15_{CPS}/16_{CPS}$; see below and fig. 1). The data matrices with the alleles retained for each individual, without any positive evidence for recombination, were used for subsequent analyses.

Different measures of nuclear genetic polymorphism of unisexual species were generally higher than the corresponding values for any representation of bisexual species (table 4), and these differences were always significant between the groups of unisexual and bisexual taxa (table 5). The average number of observed alleles for nuclear loci in unisexual species was 5.60 (SD = 2.97), versus 2.05 (SD = 1.26; or 2.52 ± 1.47, considering only data from cloning experiments) for bisexual species, and identical trends were observed for the other measures of polymorphism, with a marked difference relative to nucleotide diversity (0.00892 ± 0.00516 in unisexual vs. 0.00276 ± 0.00301 in bisexual species). Conversely, mtDNA did not show any marked trend for these parameters, if not the opposed tendency (tables 3, 4).

Table 2: Individual genotypes for the three nuclear loci investigated in unisexual species of *Calligrapha*

Species, individual	*N*	*CPS* genotype	*N*	*HARS* genotype	*N*	*Wg* genotype
C. alnicola:						
250	13	2_{AC}[62%] : 3_{AC}[38%]	12	1_{A}[100%]	19	1_{A}[79%] : 14_{c}[21%]
563	10	3_{AC}[100%]	9	1_{A}[89%] : 2_{a}[11%]	12	1_{A}[50%] : 4_{a}[8%] : 6_{a}[42%]
C169	12	1_{A}[50%] : 2_{AC}[50%]	18	1_{A}[100%]	14	6_{a}[86%] : 12_{c}[14%]
C275	18	3_{AC}[28%] : 8_{C}[39%] : 9_{c}[11%] : 13_{I}[22%]	7	1_{A}[29%] : 7_{C}[71%]	9	1_{A}[22%] : 3_{A}[33%] : 16_{I}[45%]
C. apicalis:						
552	11	8_{C}[82%] : 10_{c}[9%] : 43_{x}[9%]	8	7_{C}[75%] : 9_{I}[25%]	17	1_{A}[24%] : 13_{c}[12%] : 16_{I}[35%] : 17_{M}[29%]
C276	4	8_{c}[50%] : 13_{i}[25%] : 17_{m}[25%]	13	7_{C}[31%] : 9_{I}[46%] : 13_{M}[23%]	19	1_{A}[16%] : 16_{I}[47%] : 17_{M}[37%]
C. suturella:						
129	18	12_{I}[22%] : 17_{M}[78%]	16	12_{i}[31%] : 13_{M}[14%] : 17_{m}[56%]	13	16_{I}[54%] : 17_{M}[46%]
130	10	12_{I}[30%] : 17_{M}[70%]	12	12_{i}[25%] : 13_{M}[33%] : 17_{m}[42%]	10	16_{I}[40%] : 17_{M}[60%]
331	19	12_{I}[21%] : 17_{M}[53%] : 40_{x}[5%] : 41_{x}[21%]	15	12_{i}[27%] : 13_{M}[33%] : 17_{m}[33%] : 30_{i}[7%]	12	16_{I}[50%] : 17_{M}[50%]
333	10	14_{i}[20%] : 17_{M}[80%]	18	11_{i}[28%] : 13_{M}[39%] : 18_{m}[33%]	14	16_{I}[14%] : 17_{M}[43%] : 19_{m}[43%]
334	9	12_{I}[11%] : 21_{m}[78%] : 39_{m}[11%]	13	12_{i}[54%] : 13_{M}[15%] : 17_{m}[31%]	15	16_{I}[7%] : 17_{M}[93%]
335	13	12_{I}[31%] : 17_{M}[38%] : 41_{x}[31%]	13	12_{i}[31%] : 13_{M}[54%] : 17_{m}[15%]	12	16_{I}[33%] : 17_{M}[67%]
555	6	17_{M}[83%] : 19_{m}[17%]	7	11_{i}[29%] : 13_{M}[57%] : 18_{m}[14%]	12	16_{I}[50%] : 17_{M}[42%] : 19_{m}[8%]
C102	13	12_{I}[8%] : 15_{i}[8%] : 16_{i}[8%] : 17_{M}[61%] : 38_{m}[15%]	16	12_{i}[44%] : 13_{M}[25%] : 17_{m}[31%]	19	16_{I}[26%] : 17_{M}[74%]
C103	14	14_{I}[7%] : 17_{M}[93%]	18	11_{i}[28%] : 13_{M}[33%] : 16_{M}[39%]	15	16_{I}[40%] : 20_{m}[60%]
C104	9	17_{M}[78%] : 22_{m}[22%]	14	11_{i}[50%] : 13_{M}[14%] : 16_{M}[36%]	16	16_{I}[37%] : 20_{m}[63%]
C. vicina:						
C376	14	24_{p}[50%] : 25_{P}[29%] : 29_{PR}[21%]	15	19_{P}[47%] : 23_{R}[20%] : 29_{x}[33%]	14	25_{P}[21%] : 26_{P}[29%] : 31_{R}[50%]
C. virginea:						
C135	6	4_{ST}[33%] : 13_{I}[50%] : 42_{st}[17%]	16	4_{S}[38%] : 6_{s}[25%] : 10_{I}[37%]	13	7_{ST}[23%] : 16_{I}[77%]
C195	12	5_{S}[25%] : 6_{st}[50%] : 12_{I}[25%]	10	4_{S}[70%] : 5_{S}[10%] : 9_{I}[20%]	13	7_{ST}[38%] : 16_{I}[62%]

Note: Shown are the number of analyzed clones (*N*) and their respective cloning proportions, as well as an indication of their phylogenetic proximity to genetic variants in bisexual species as members of the most derived highly supported clade (see fig. A1, available online). Allele numbers correspond to these in figure 1. Alleles shared (indicated with capital letters) or genetically close (lowercase) to those in bisexual taxa: A/a = *C. alni*; C/c = *C. confluens*; I/i = *C. ignota*; M/m = *C. multipunctata*; P/p = *C. philadelphica*; R/r = *C. rowena*; S/s = *C. scalaris*; T/t = *C. amator*; x = unknown. *CPS* = carbamoyl phosphate synthase; *HARS* = histidyl-tRNA synthetase; *Wg* = wingless.

Table 3: Individual genotypes for the three nuclear loci investigated in bisexual species of *Calligrapha*

Species, individual	*CPS* genotype	*HARS* genotype	*Wg* genotype
C. alni:			
C128	*1*	*1*	*1*:*5*
116	*2*	*1*	*1*
126	*3*	*1*	*1*:*2*:*3*
C. amator:			
247	*4*	*3*	*7*:*8*
C. confluens:			
121	*2*:*7*	*7*	*10*
123	*3*:*8*	*7*	*10*:*11*
C. floridana:			
C318	*11*	*8*	*15*
C. ignota:			
556	*12*	*9*:*10*	*16*
C164	*12*:*13*	*9*	*16*
C. multipunctata:			
336	*17*	*13*:*15*	*17*:*18*
C027	*17*	*13*	*17*
C031	*17*:*18*	*13*:*14*	*17*
C041	*17*:*18*	*13*	*21*:*22*
CM064	*17*:*20*	*13*:*16*	*17*
C. philadelphica:			
283	*27*:*28*	*19*	*27*
C072	*28*	*19*	*25*
C091	*25*:*26*	*19*	*23*:*24*
C100	*27*:*28*	*19*	*23*
C182	*23*:*29*	*19*	*26*:*27*
C500	*27*	*19*	*23*
C530	*27*	*19*	*23*
C. pruni:			
C133	*32*	*20*:*21*	*28*:*29*
C140	*30*:*31*	*20*	*28*:*29*
C. rhoda:			
C163	*33*:*34*	*22*	*30*
C. rowena:			
C411	*29*	*23*:*24*	*31*
C547	*35*	*23*	*31*
C. scalaris:			
405	*4*	*5*	*9*
C326	*5*	*4*	*7*
C. spiraea:			
114	*36*	*25*:*26*	*32*
C. verrucosa:			
C066	*37*	*27*:*28*	*33*

Note: Allele numbers correspond to these in figure 1. *CPS* = carbamoyl phosphate synthase; *HARS* = histidyl-tRNA synthetase; *Wg* = wingless.

Nuclear Gene Genealogies

Figure 1 shows the allele genealogies for the three investigated nuclear loci after objective exclusion of chimeras. For species with more than one allele sampled, there were very few cases of species monophyly for any of the data sets, that is, groups of alleles from a single species connected to the remainder of the network by a single branch. For the *CPS* locus, only the bisexual *C. pruni* and *C. rhoda* satisfied this condition, and for *HARS*, only the bisexual *C. pruni*, *C. spiraea*, and *C. verrucosa* satisfied it; for *Wg*, there was no such group. Species monophyly was challenged either by allele sharing or close phylogenetic proximity between two or more species or by alleles from the same species dispersed on the genealogy (and with alleles from other species in their connecting paths). There was an extensive pattern of allele sharing for each locus between unisexual and bisexual species of *Calligrapha*, showing remarkable similarities across loci (fig. 1). The unisexual *C. apicalis* shared genetic variants with up to four bisexual species for the whole data set, and *C. ignota* stood out among bisexual species for sharing alleles with all unisexual species except *C. vicina*. Allele sharing between bisexual species was exceptional and affected only closely related species as recognized from a morphological point of view and/or with similar ecologies, that is, *C. alni* with *C. confluens* and *C. philadelphica* with *C. rowena* for *CPS*, and *C. amator* with *C. scalaris* for both *CPS* and *Wg* (fig. 1). Figure 2 shows the frequency distribution of mutational steps between alleles in uni- and bisexual species, with an apparent trend of the former species to present higher allele dispersion in the respective networks for each marker. The pattern was less clear for *Wg* due to one very divergent allele in one of the specimens of *C. alni* producing mutation counts in the same range as the highest values observed in some unisexual species (with a consistently high π value; table 4). Conversely, the apparent allele dispersion of *C. alnicola* is associated with a single specimen of the four exemplars classified with this name. This species is difficult to recognize due to marked morphological similarity with some of the variation observed in *C. alni*. Potential misidentifications in this case would make some of our estimates more conservative, smoothing differences between unisexual and bisexual parameters, for instance, for some measures of genetic polymorphism or inflating the observed frequencies of low allele divergences in unisexual taxa.

Times to Coalescence

Each locus investigated in *Calligrapha* fitted a different evolutionary model: HKY85+G in the case of *CPS*, HKY85+I for *HARS*, and GTR+G+I for *Wg*. Adjusting for the locus-specific substitution profiles and the range of clock (SC, ULN) and diversification (CSc, LGc, BSc, Ysp, BDsp) models tested showed similar results and the same trends across loci and taxa (fig. A2; figs. A1, A2 are available online). Figure 3 shows the summary of results

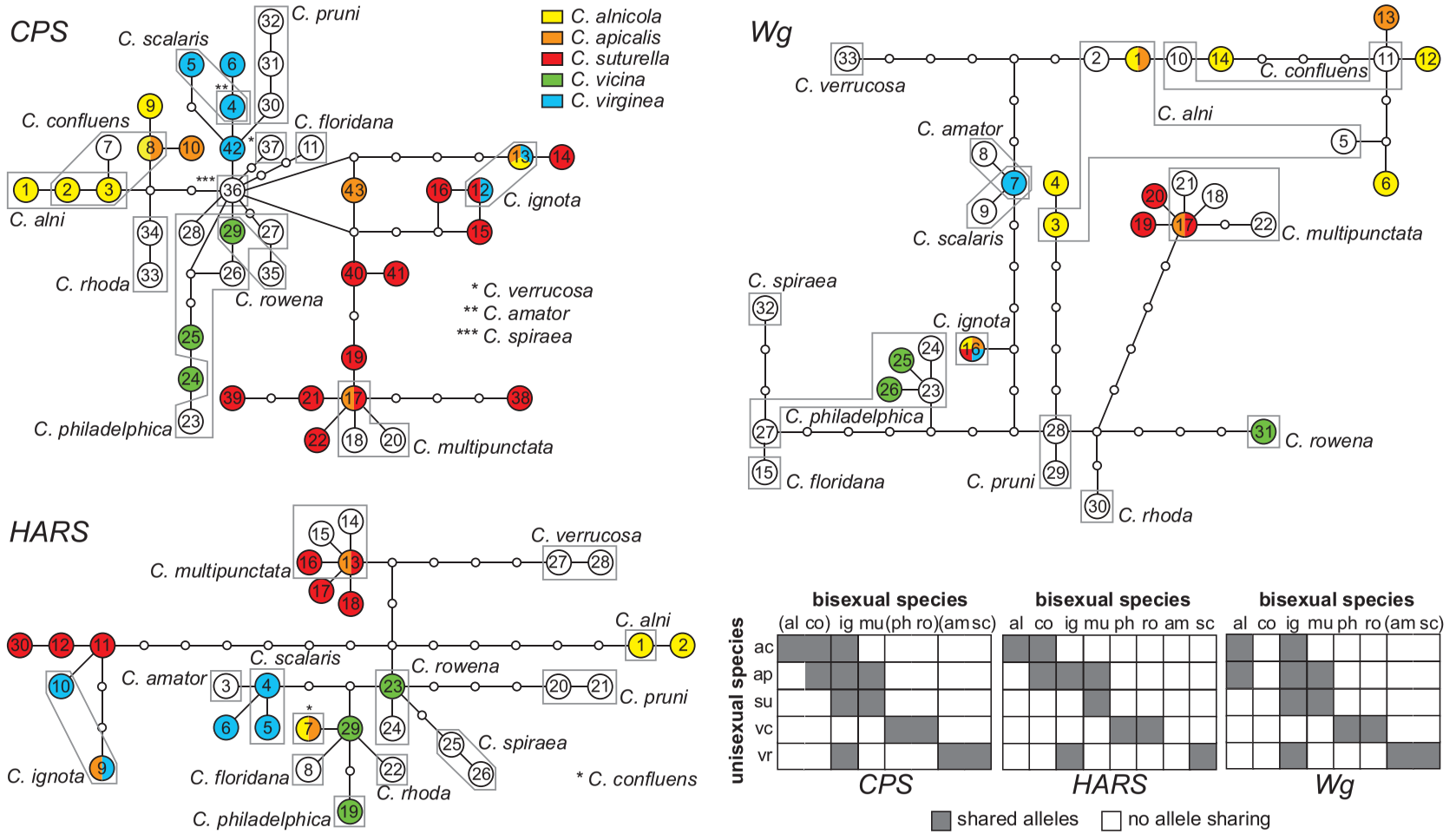

Figure 1: Statistical parsimony networks of allele diversity for the carbamoyl phosphate synthase (*CPS*), histidyl-tRNA synthetase (*HARS*), and wingless (*Wg*) loci in *Calligrapha*. Color codes identify genetic variants retrieved from unisexual species, and gray polygons group those retrieved in bisexual species, identified by name. Each allele is identified with a number, as in table 1. *Bottom right*, pattern of allele sharing between unisexual and bisexual species of *Calligrapha* by gene. Unisexual taxa: *C. alnicola* (ac), *C. apicalis* (ap), *C. suturella* (su), *C. vicina* (vc), and *C. virginea* (vr); bisexual taxa: *C. alni* (al), *C. amator* (am), *C. confluens* (co), *C. ignota* (ig), *C. multipunctata* (mu), *C. philadelphica* (ph), *C. rowena* (ro), and *C. scalaris* (sc).

Table 4: Genetic polymorphism of carbamoyl phosphate synthase (*CPS*), histidyl-tRNA synthetase (*HARS*), and wingless (*Wg*) loci in different species of *Calligrapha* with alternative reproductive modes

Species	N	h_{CPS}	S_{CPS}	π_{CPS}	h_{HARS}	S_{HARS}	π_{HARS}	h_{Wg}	S_{Wg}	π_{Wg}	h_{cox1}	S_{cox1}	π_{cox1}
Unisexual:													
C. alnicola	4	6	12	.00451	6	11	.00408	7	18	.01709	2	1	.00068
C. apicalis	2	5	13	.00746	5	17	.01068	4	20	.02176	1	...	...
C. suturella	10	12	20	.00708	12	14	.00634	4	11	.01136	2	1	.00033
C. vicina	1	3	5	.00444	3	4	.00297	3	8	.01282	1	...	...
C. virginea	2	6	14	.00701	6	16	.00860	2	5	.00754	2	6	.00726
Bisexual:													
C. alni	3	3	2	.00178	1	...	...	4	14	.01207	3	52	.04691
C. amator	1	1	...	...	1	...	...	2	1	.00226	1	...	...
C. confluens	2	4	4	.00266	1	...	...	2	5	.00754	2	3	.00406
C. floridana[a]	1	1	...	...	1	...	...	1	...	...	1	...	...
C. ignota	2	2	8	.00710	2	4	.00297	1	...	...	2	1	.00135
C. multipunctata	5	3	2	.00090	4	3	.00083	4	4	.00259	5	10	.00694
C. philadelphica	7	6	9	.00431	1	...	...	5	8	.00591	6	42	.02101
C. pruni[a]	2	3	2	.00178	2	1	.00074	2	1	.00151	1	...	...
C. rhoda[a]	1	2	1	.00133	1	...	...	1	...	...	1	...	...
C. rowena[a]	2	2	4	.00533	2	1	.00074	1	...	...	1	...	...
C. scalaris	2	2	3	.00399	2	1	.00111	2	1	.00226	2	13	.01759
C. spiraea[a]	1	1	...	...	2	1	.00111	1	...	...	1	...	...
C. verrucosa[a]	1	1	...	...	2	1	.00111	1	...	...	1	...	...

Note: Data for a mitochondrial DNA marker is given for comparative purposes. N = number of individuals analyzed; h = number of individual alleles; S = number of segregating sites; π = nucleotide diversity.

[a] Species not cloned.

for two such tested scenarios, representative of extremes in analytical conditions, namely, a constant-size coalescent diversification under a strict clock and Yule speciation under uncorrelated lognormal clock relaxation. Age intervals for the most recent common ancestor of unisexual species were usually and consistently deep (more than 2.0 Ma), particularly for diversification models accounting for species diversification. Conversely, the corresponding times for bisexual species were generally shallower (less than 2.0 Ma) and more erratic in behavior, that is, different loci showed opposing trends in different taxa.

Mitochondrial Phylogeny

MtDNA data was partitioned according to their noncoding and protein-coding natures, with the latter according to codon position; an HKY+I model was objectively selected for first codon positions, second codon positions of *cox1*, and for noncoding markers (these also with heterogeneity in rates of substitution, G), while a GTR model fitted best to second codon positions in *cox2* and third codon positions (the latter with I+G parameters). The Bayesian tree for concatenated mtDNA markers (fig. 4) showed several interesting features, some already described in a previous phylogenetic study of *Calligrapha* (Gómez-Zurita et al. 2006). Most species sampled for more than one individual appeared as paraphyletic or distantly polyphyletic, with the exception of *C. confluens*, *C. floridana*, *C. ignota*, and *C. pruni* among the bisexual species and *C. alnicola* (but see below), *C. apicalis*, and *C. virginea* among the unisexual. The most remarkable observation on the phylogeny was the existence of a trichotomy near the base of the tree giving rise to an exclusively bisexual (B) clade and two unisexual clades, one with *C. alnicola* (U1) and one with the remaining unisexual taxa (U2). Both U1 and U2 included representatives of bisexual species, possibly *C. alni* and *C. philadelphica*/*C. rowena*, respectively, which, based on the incongruent signal from nuclear loci, could be interpreted as individuals with the same introgressed mtDNA as present in unisexual species. Despite lack of support, data were compatible with a single old evolutionary origin of the mitochondrial diversity observed in all unisexual species or at most two from the same period.

Discussion

Unisexual Calligrapha *are Hybrid Genomic Mosaics*

In an earlier phylogenetic attempt to explain the origins of unisexual species in *Calligrapha*, we found a trend linking unisexuality in this genus with phylogenetic incongruence between mtDNA and nuclear markers (Gómez-Zurita et al. 2006). This pattern was suggestive of an interspecific hybrid origin for these lineages, but the con-

Table 5: Results of Mann-Whitney tests for differences in median values of several diversity parameters estimated in unisexual and bisexual samples of *Calligrapha* and for different loci

Locus, parameter	Mann-Whitney *U*	Wilcoxon *W*	*Z*	*P* (two-tailed)	*P* (one-tailed)
CPS:					
h	5.5	96.5	−2.709	.007	.004
S	2	47	−2.748	.006	.004
π	6	51	−2.202	.028	.029
HARS:					
h	1	92	−3.225	.001	.000
S	.5	28.5	−2.868	.004	.003
π	.5	28.5	−2.790	.005	.003
Wg:					
h	12	103	−2.092	.036	.046
S	5	33	−2.052	.040	.048
π	2.5	30.5	−2.445	.015	.010
cox1:					
h	32.5	123.5	0	1.000	1.000
S	3	9	−1.576	.115	.167
π	3	9	−1.549	.121	.167

Note: *CPS* = carbamoyl phosphate synthase; *h* = number of individual alleles; *S* = number of segregating sites; π = nucleotide diversity; *HARS* = histidyl-tRNA synthetase; *Wg* = wingless.

firmation required a fundamental look into the genome of these species to test the hypothesis that hybrid lineages should show some degree of genetic admixture. Even in the case of genomes of documented hybrid origin, this evidence may affect only a small proportion of adaptive genes, as empirically found in *Heliconius* butterflies (Heliconius Genome Consortium 2012), up to the theoretical expectation of an alloploid hybrid lineage, where each parental genome remains intact, as in the F_1 individuals of a successful interspecific mating or even in their descendants if recombination is impaired (Abbott et al. 2013). Our ignorance about genome structure and the degree of potential admixture in *Calligrapha* called for a blind, exploratory strategy as followed here, sequentially testing single-copy nuclear loci for which no linkage expectations existed, anticipating that at least some would show the expected signature of admixture.

The strong prevalence of chimeric PCR artifacts in unisexual individuals was already an indication that these DNA extractions included divergent copies of the target locus and were thus prone to priming by incomplete homologous but divergent PCR products. Moreover, objective filtering out of these artifacts generated a data set that was compatible with expectations based on the ploidy levels of the species involved (tables 2, 3), and measures of genetic diversity revealed a consistent pattern of higher diversity associated with unisexual species (fig. 2; table 4). Finally, from a genealogical viewpoint, this genetic diversity showed that their phylogenetic distribution (fig. 1) and coalescent dynamics (fig. 3) were also compatible with interspecific divergence and evolutionary processes involving more than one species.

Two major processes are typically invoked to explain species paraphyly and polyphyly in gene trees (assuming that paralogy is not an issue): incomplete lineage sorting and hybridization (Funk and Omland 2003). Both processes can occur together and are difficult to tease apart, especially in cases of recent or ongoing hybridization. Although several analytical approaches have been proposed, no methodology yet allows for their reliable distinction (Joly et al. 2009). Even worse, no method exists to deal with complex systems like *Calligrapha*, where the ideally suited multispecies coalescent method is impracticable given the lack of a species tree to anchor gene tree evolution (Degnan and Rosenberg 2009) and because alternative ploidy conditions and reproductive modes (hence, alternative coalescent dynamics) coexist in the same evolutionary tree. However, we can exploit the idea that incomplete lineage sorting is fundamentally a random process and thus expected to generate random patterns. Yet our data show up to three distinctive nonrandom patterns and are therefore generally consistent with a deterministic process—hybridization—being at play in this system. Allele dispersion in the gene trees affects all unisexual species and genes, while it is only rampant for *C. alni* in the case of the *Wg* gene and perhaps *C. ignota* for *CPS* among bisexual species. Also, the estimated divergence times for all unisexual taxa are consistently deeper than most equivalent estimates for bisexual species, especially when a realistic diversification model is implemented. The few ex-

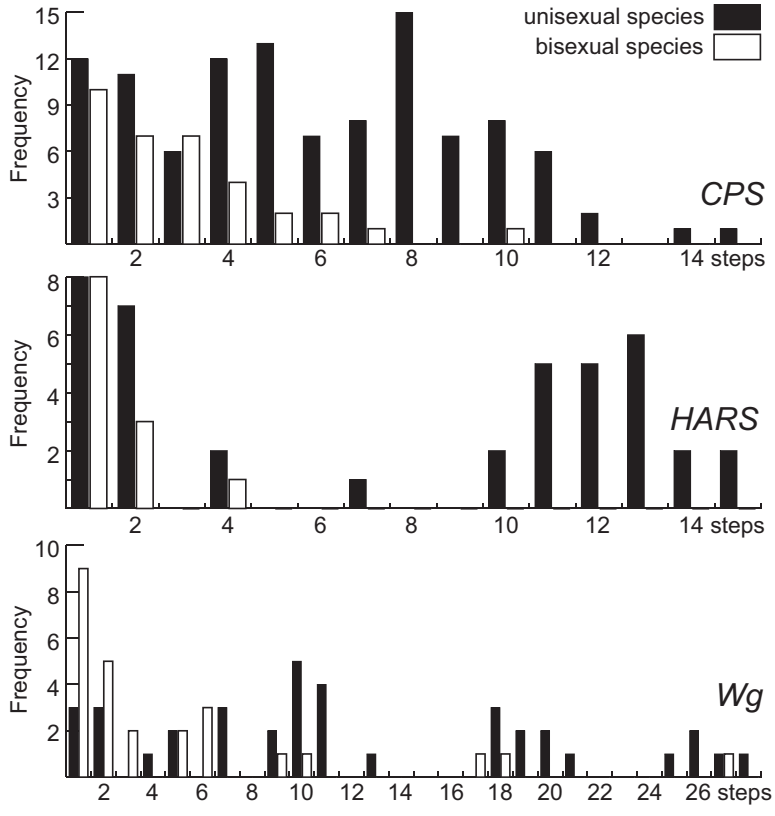

Figure 2: Frequency distribution of intraspecific genetic distances for each locus analyzed in *Calligrapha* and expressed as number of mutational differences between alleles, distinguishing between unisexual and bisexual species. *CPS* = carbamoyl phosphate synthase; *HARS* = histidyl-tRNA synthetase; *Wg* = wingless.

ceptions found for these two patterns could nonetheless reflect the stochastic nature of incomplete lineage sorting, since this process is not mutually exclusive with hybridization (Joly et al. 2009). But there is an additional nonstochastic pattern providing unequivocal confirmation of interspecific hybridization associated with unisexual *Calligrapha*. Indeed, unisexual species show remarkably concordant patterns of allele sharing with bisexual counterparts for all assayed genes, an extraordinarily unlikely outcome for unlinked loci if these patterns were the result of a random assortment of genome blocks in the diversification of this group. There are millions of theoretically possible allele-sharing patterns for the contingency matrices shown in figure 1, yet independent genes repeat essentially the same pattern, with differences explainable by diagnosis problems for three pairs of bisexual taxa and sampling effects.

Refining the Previous Evolutionary Model of Unisexuality in Calligrapha

A previous attempt to explain the evolutionary origins of unisexual *Calligrapha* by Gómez-Zurita et al. (2006) used direct sequencing of PCR products for an intergenic spacer of the nuclear ribosomal RNA gene cluster (*ITS2*) and elongation factor 1-alpha. These data were compatible with a single parental donor of nuclear variation for unisexual species, different from the lineage contributing their mtDNA. Multiparental origin of the unisexual genomes possibly remained undetected given that neither cloning nor phasing of nucleotide polymorphisms was used. However, the possibility that mosaicism does not extend to the entire genome should not be neglected either (although it generally applies to homoploid hybrids only; Abbott et al. 2013), and while it shows for the three markers used here, it may have been purged for the others. Indeed, a pilot cloning study concerning *ITS2* alone, where 126 *ITS2* clones were sequenced from two *C. suturella* specimens (IBE-JGZ-0333 and IBE-JGZ-0334, both used here), showed a single *ITS2* family, indistinguishable from 30 clones of two specimens of *C. multipunctata* and different from any other available *Calligrapha ITS2* sequences (data not shown). In this case, even if concerted evolution could be hampered by the lack of recombination (Waters and Schaal 1996; Campbell et al. 1997), it is possible that it

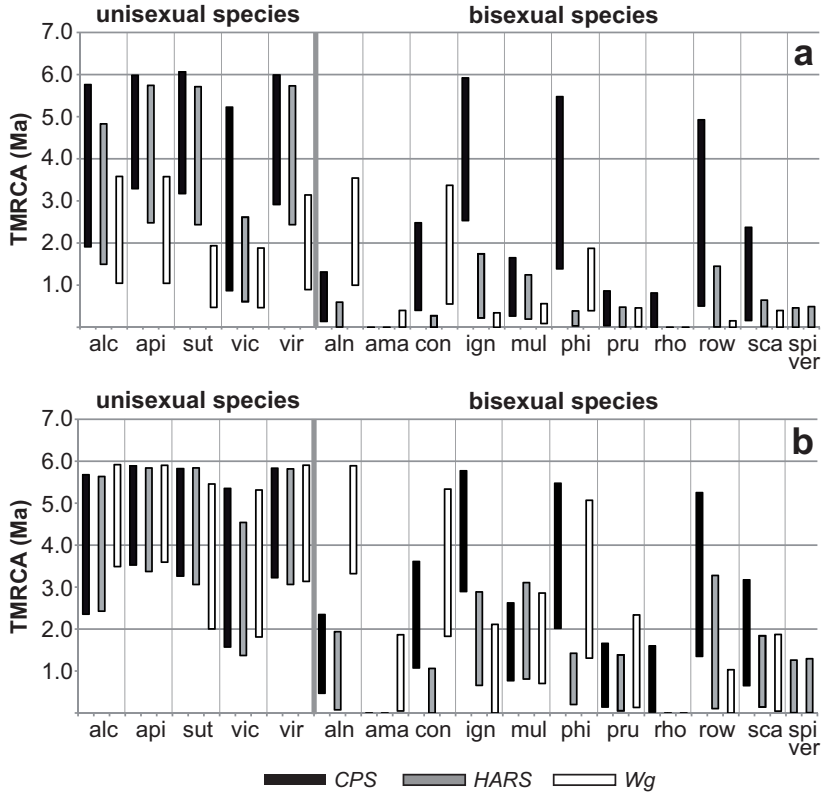

Figure 3: Two examples of results from the time for the most recent common ancestors (TMRCA) test for unisexual and bisexual species of *Calligrapha*: constant size coalescent under a strict clock (*a*) and Yule speciation under an uncorrelated lognormal relaxed clock (*b*). (Results from the remaining models tested are in fig. A2, available online.) Bars represent the 95% highest posterior density confidence intervals expressed in millions of years (Ma) for the depth of coalescence of alleles observed in every species polymorphic for a given locus (black = carbamoyl phosphate synthase [*CPS*], gray = histidyl-tRNA synthetase [*HARS*], white = wingless [*Wg*]). Unisexual taxa: *C. alnicola* (alc), *C. apicalis* (api), *C. suturella* (sut), *C. vicina* (vic), and *C. virginea* (vir); bisexual taxa: *C. alni* (aln), *C. amator* (ama), *C. confluens* (con), *C. ignota* (ign), *C. multipunctata* (mul), *C. philadelphica* (phi), *C. pruni* (pru), *C. rhoda* (rho), *C. rowena* (row), *C scalaris* (sca), *C. spiraea* (spi), and *C. verrucosa* (ver).

had time to effectively homogenize this multigene family before bisexuality and recombination ceased. Alternatively, it may have continued to operate in supposedly clonal organisms, as has been suggested for *Daphnia* water fleas (Crease and Lynch 1991), *Heteronotia* geckos (Hillis et al. 1991), and *Acanthoxyla* stick insects (Morgan-Richards and Trewick 2005). In any case, these results caution against the use of multigene families undergoing concerted evolution to investigate evolution and parentage of unisexual species or the use of systems with alternative reproductive modes to investigate the evolutionary dynamics of these genes in the hopes of disentangling the effects of concerted evolution.

The hybridization model originally proposed for *Calligrapha* was simple, merely considering two hybridizing species (a single distinctive parental for each nuclear and mitochondrial genome). This model struggled to explain the loss of one of the parental nuclear genomes, proposing instead that preferential polyploidization of one of the genomes and/or PCR biases hindered the detection of both parental genomes (Gómez-Zurita et al. 2006). With the new data available, our current understanding of this evolutionary mechanism has changed quite radically.

First of all, we have included here a few additional bisexual *Calligrapha* and two new unisexual species (the sample from Ottawa named previously as *C. vicina*, IBE-JGZ-0283, was misidentified, and the specimen belongs to the bisexual *C. philadelphica* species complex; table 1). Our expanded data set shows that mtDNA from all unisexual species effectively form a clade with a few specimens currently assigned to otherwise polyphyletic bisexual taxa, which may have introgressed the mtDNA associated with unisexuality. This is compatible with a single (or two, at most) old evolutionary lineage (~3 Ma; Gómez-Zurita et al. 2006) acting as donor of the mtDNA present in all the unisexual species and divergent from any of those present

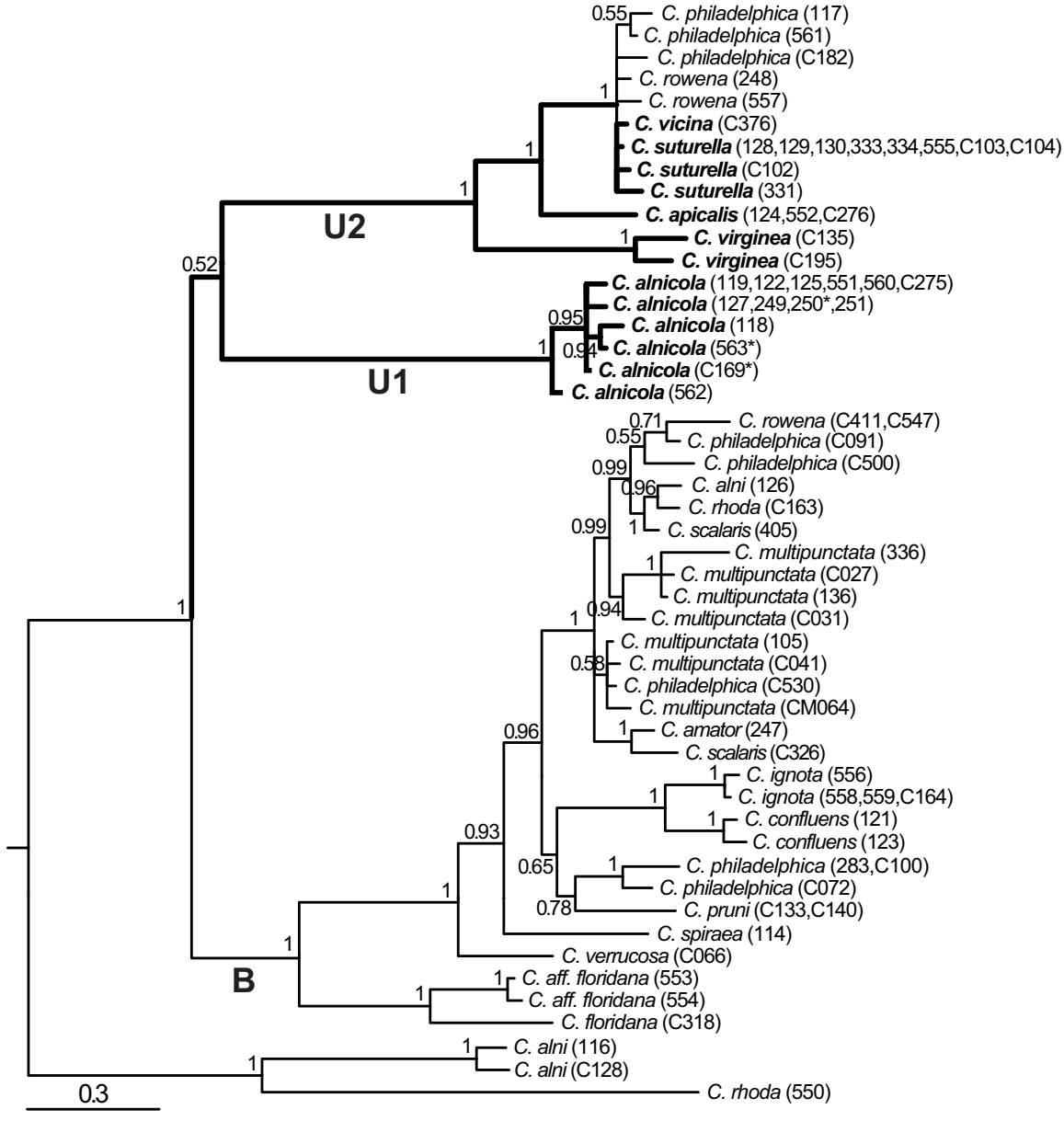

Figure 4: Mitochondrial DNA (mtDNA) haplotype phylogeny of North American *Calligrapha* based on Bayesian inference of partial sequences for cytochrome *c* oxidase subunits 1 and 2, *cox1* and *cox2*, and the large ribosomal RNA subunit, *rrnL*, showing posterior probabilities >0.50 next to their respective node. The tree includes genetic data from the current study and Gómez-Zurita et al. (2006; see table A1, available online) and is rooted with *C. dislocata* (removed secondarily); thicker lines highlight the branches leading to haplotypes present in unisexual taxa. The main bisexual (B) and unisexual (U1 and U2) evolutionary lineages are identified as well as the individuals carrying each haplotype, in brackets. Individuals of *C. alnicola* marked with an asterisk are discussed in the main text as presenting nuclear genotypes consistent with their belonging to *C. alni* but with the same introgressed mtDNA as *C. alnicola*.

in the currently studied bisexual taxa (with some exceptions; see next section). Nuclear markers, in contrast, enable us to relate in every case unisexual genotypes with extant donors of allelic diversity (fig. 1; table 2). The second notable difference with our previous model is that, here, two but usually more *Calligrapha* species among a reduced number of potential candidates may have participated sequentially in the establishment of every hybrid unisexual lineage. Thus, ignoring the contribution of the unknown source of mtDNA, among the 17 North American species capable in theory of interspecific hybridization, six or at most eight have potentially contributed to

unisexual diversity (there is lack of resolution to distinguish among genomic pools of *C. alni* and *C. confluens*, both alder specialists, and of *C. amator* and *C. scalaris*, both associated with *Tilia* and, for the latter, *Ulmus*).

Evolutionary Roads to Extant Unisexual Diversity in Calligrapha

In every unisexual case, the patterns of allele sharing with extant bisexual *Calligrapha* allow us to recognize the unisexual species' parentals or the parental lineages of their nuclear genomes. While merely a rough guide, the proportion of clones from each putative parental (and, to some extent, the actual number of alleles retrieved per individual; tables 2, 3) can be tentatively used to hypothesize the genomic makeup for these species. The simplest scenario involves *C. philadelphica* (*P* genome) and *C. rowena* (*R* genome), both feeding on *Cornus*, acting as parentals of the unisexual *C. vicina* (also a dogwood specialist). Cloning proportions for this tetraploid species (Robertson 1966) are suggestive of either an excess of *P* genomes (assuming that they are representative of genomic constitution) or a similar genomic contribution from both species (*PPPR* or *PPRR*). In this case, mtDNA also could have been received from any of these species via introgression in the parentals, since very similar variants occur in the three species (fig. 4).

The origins of *C. alnicola*, *C. suturella*, and *C. virginea* involved interspecific crossings of *C. ignota* (*I* genome) with *C. alni*/*C. confluens* (*A*/*C* genomes), *C. multipunctata* (*M* genome), or *C. amator*/*C. scalaris*, respectively. Three of four specimens assigned to *C. alnicola* did not produce data compatible with their being mosaics; instead, they bear genetic variants associated with or closer to these in *C. alni* (both species are phenotypically similar and different from the very characteristic *C. confluens*). We can reinterpret our data recognizing these specimens as slightly anatomically distinctive *C. alni* with the introgressed mtDNA associated with unisexuality. The situation would then be analogous to the case of *C. vicina* and provide a route for the noncanonical mtDNA to reach *C. alnicola* in the evolutionary pathway to this species. The specimen of *C. alnicola* providing clear-cut evidence for interspecific hybridization (IBE-JGZ-C275) yielded cloning data with a higher quantitative contribution from *C. alni* and/or *C. confluens* (e.g., 100% of *HARS* clones), relative to the unrelated *C. ignota*. *Calligrapha alnicola* is tetraploid (Robertson 1966); hence, we predict an *AAAI* or *CCCI* genomic makeup for this species. Nonetheless, *AACI* or *ACCI* also offer plausible hypotheses due to a lack of resolution between *C. alni* and *C. confluens* with our markers. These scenarios would imply the hybridization of *C. ignota* with putative hybrids between *C. alni* and *C. confluens*, a possibility hinted by *HARS* data and seemingly feasible in this system (as in *C. apicalis*; see below), but it is a hypothesis that needs testing. *Calligrapha alnicola*, *C. alni*, and *C. confluens* share food choice of *Alnus* but not with *C. ignota*. The prevalence of genomic contribution from *Alnus*-feeding species in the unisexual polyploid hybrid may override the ecological conflict caused by divergently adapted genes, in this case, related to trophic preference (Lynch 1984). However, even though dosage imbalance seems a plausible hypothesis, the mechanisms explaining genome dominance are not yet fully understood (Doyle et al. 2008). *Calligrapha* stands out as a promising system to investigate them.

The evolutionary pathway to *C. suturella* involved the recent hybridization between *C. ignota* and *C. multipunctata*. *Calligrapha suturella* was tested with the highest number of specimens and clones, and it again shows a consistent lower proportion of *C. ignota* alleles relative to *C. multipunctata*. This is the only unisexual species lacking cytological information, although we assume that it is tetraploid like other unisexual *Calligrapha* (Robertson 1966). Intriguingly, most specimens and markers yielded two (46.7%) or three (43.3%) alleles, suggestive of triploidy, with a single specimen (IBE-JGZ-0331) and marker (*HARS*) producing data compatible with tetraploidy (this specimen had four alleles and IBE-JGZ-C102 five alleles for *CPS*, in both cases with at least two alleles very likely unpurged chimeras, judging from their positions and association to loops in the *CPS* genealogy; fig. 1). Regardless of the ploidy level, the genomic constitution of *C. suturella* seems to include, roughly derived from cloning frequencies, a single *C. ignota* genome (*MMI* or *MMMI*). This, in turn, suggests backcrossing of the interspecific hybrid with *C. multipunctata*, very much as in the original model (Gómez-Zurita et al. 2006) but with the complicating issue of an unknown donor of mtDNA shared by all *C. suturella* specimens that could be either a third species or an introgressed *C. multipunctata* female. As before, the asymmetric genomic mosaic supports ecological and phenotypic similarities of the willow-feeding *C. suturella* with only one parental species, specifically, the willow-feeding *C. multipunctata* (Gómez-Zurita et al. 2004).

Tetraploid unisexual *C. virginea* is a genetic mosaic between *C. ignota* and a member of the *C. scalaris* complex (Brown 1945). The exploratory analysis of genomic contributions is more ambiguous than in previous examples, with *CPS* and *HARS* pointing at a major contribution of *C. amator*/*C. scalaris* and *Wg* indicating *C. ignota*. Based on the prediction of quantitative effects of genomic contribution on the phenotypic resemblance of hybrids to their parentals, we can hypothesize again a lower input from *C. ignota*, since the unisexual species is nearly iden-

tical anatomically and shares hosts with the *Tilia*-adapted (or *Tilia*-tolerant) species of the *C. scalaris* complex.

Finally, *C. apicalis* has a similar pattern as the previous examples but with an additional twist. Its genetic constitution is compatible with interspecific crosses involving at least three divergent lineages of *Calligrapha*, namely, *C. alni*/*C. confluens*, *C. multipunctata*, and *C. ignota*. Clone frequencies are inconsistent between markers and individuals, except for the possible extrapolation of a single *C. multipunctata* genome based on lower frequencies. Therefore, we consider the following tetraploid alternatives: *CCIM*, *ACIM*, *AIIM*, or *CIIM*. Two nonmutually exclusive routes could explain these patterns: hybridization between individuals of the *C. alni*/*C. confluens* genomic pool, with individuals of the hybrid bisexual stage leading toward *C. suturella* (*CC*/*IM* or *AC*/*IM*), or hybridization between individuals of the two hybrid lineages leading to *C. alnicola* and *C. suturella* (*AI*/*IM* or *CI*/*IM*). The former, backcrossing of a hybrid with a third species, gains more weight considering the mtDNA phylogenetic proximity between *C. apicalis* and *C. suturella*, which implies that they share a recent common ancestor but not with *C. alnicola*. These species' ecology also supports this interpretation, combined with genome additive effects, since *C. apicalis* shares host with *C. alni* and *C. confluens*. But the second possibility, an origin based on the hybridization of hybrids, has the appeal of explaining tetraploidization in a single step by syngamy of unreduced gametes contributed by both hybrids (Alves et al. 1999). Botanical literature includes numerous examples of hybrids of hybrids, but research on animal unisexuality has also reported several cases where interspecific bisexual hybrids hybridize with a third bisexual species, giving rise to the unisexual phenotype, and this process also explains ploidy increases via unreduced gametes, typically eggs in the first class of hybrids. Classical unisexual vertebrate systems include well-known examples of these tri- and even tetramodal complex hybridization scenarios, including *Aspidoscelis* whiptail lizards (Lowe and Wright 1966), *Ambystoma* mole salamanders (Bogart et al. 2007), and *Leuciscus* (Alves et al. 2001) and *Poeciliopsis* (Mateos and Vrijenhoek 2002) fish. There are known cases among the invertebrates, too, such as stick insects in the genera *Bacillus* (Mantovani et al. 1999) and *Leptynia* (Ghiselli et al. 2007).

A Complex Model of Sequential Hybridization Leads to Unisexuality in Calligrapha

Each independent origin of unisexuality in *Calligrapha* can be considered at present unique or extremely rare by virtue of mtDNA monophyly, and these lineages, characterized by very low genetic diversities, are of recent origin or recently expanded (Gómez-Zurita et al. 2006). However, data compatible with nearly reciprocal mtDNA monophyly of unisexual and bisexual taxa and incompatible with expected species relationships based on morphology clash with the demonstration of independent evolutionary origins. Such a conflict cautions about the possibility of a selective sweep affecting the mtDNA phylogeny. This process can be driven by maternally inherited endosymbionts, such as *Wolbachia*, which can both confound mtDNA phylogenies and induce feminization or unisexuality in arthropods (Hurst and Jiggins 2005). Alternatively, the unknown mtDNA donor(s) could be an extinct species or missing from our taxonomic sampling (Avise et al. 1992; Little and Hebert 1996). Nonetheless, orphan sources of mtDNA in hybrid unisexual lineages have been reported in the literature, for example, in *Acanthoxyla* stick insects (Morgan-Richards and Trewick 2005) or *Rhopalosiphum* aphids (Delmotte et al. 2003). But regardless of mtDNA being an ill-suited guide for the species tree of *Calligrapha* or inference of parentals involved in the hybridization events, there is a rather clear emerging pattern of complex hybridization pathways in this genus, compellingly represented by *C. apicalis*, which has at least three divergent lineages contributing to its nuclear allele diversity. Based on the data and interpretations above, figure 5 outlines alternative routes to unisexuality in *Calligrapha*, with several well-defined stages.

Stage i: ancient hybridization. Based on data compatible with monophyly (or two contemporaneous events) of mtDNA present in all unisexual species and the recognition of bisexual taxa deeply polymorphic for mtDNA (e.g., *C. alni*, *C. philadelphica*, and *C. rowena*), we hypothesize that by the end of the Pliocene one possibly extinct species of *Calligrapha* hybridized with several others in ancestral lineages of extant bisexual species.

Stage ii: backcrossing and mtDNA introgression. Backcrossing of these hybrids with one of their respective parental populations in successive generations (perhaps for a time span of 2 Ma) led to the introgression of this mtDNA within a xenobiotic genomic background, homogeneous with the rest of conspecifics, as well as a stable mtDNA polymorphism observed in several bisexual species today. Phylogenetic concentration of unisexuality in the mtDNA tree is suggestive that these lineages also received a factor (or preadaptation) that would trigger the origin of unisexuality in the right circumstances.

Stage iii: recent hybridization. A second wave of interspecific hybridization events took place from the middle to late Pleistocene (Gómez-Zurita et al. 2006), perhaps motivated by range shifts associated to changes in climatic conditions (Hewitt 1996, 2000; Kearney 2005). Judging from field observations, the recognition of somewhat intermediate phenotypes in collection specimens of *Calligrapha*, and literature reports (Robertson 1966), interspe-

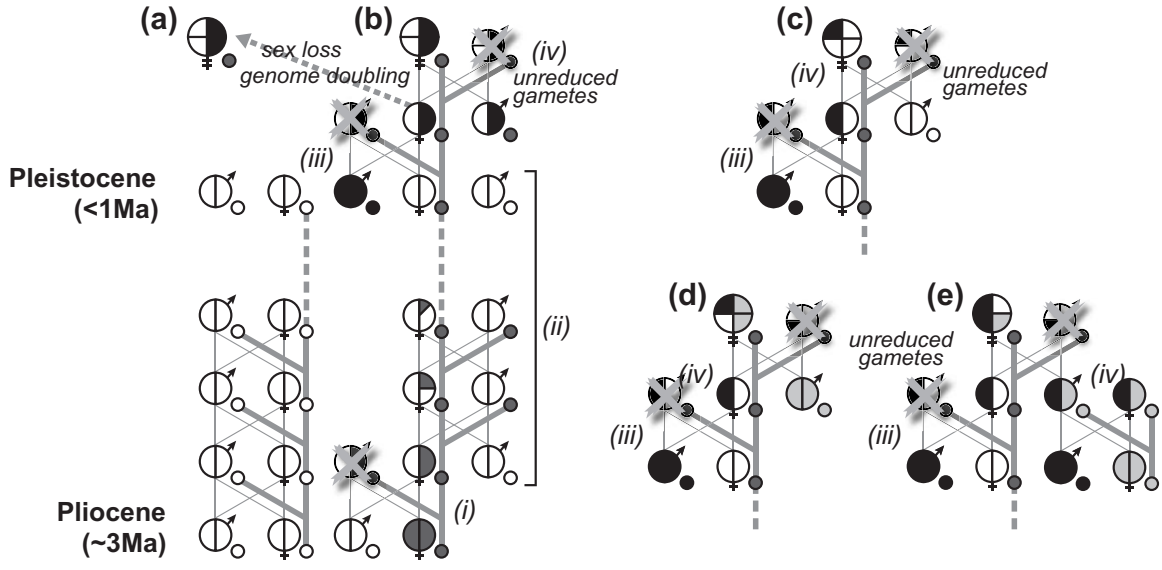

Figure 5: Sequential hybridization model explaining the evolution of unisexual species in *Calligrapha*. Male and female nuclear genomes are identified with the appropriate symbols, a small circle represents their mitochondria/mitochondrial DNA (mtDNA), and different shades from white to black indicate their taxonomic differences; fine gray lines represent sex between generations (meiosis and syngamy), and thick gray lines represent clonal, vertical maternal transmission of the mtDNA genome; a crossed symbol represents the purging effects on hybrid males associated to Haldane's rule. *(i)*, A single or at most two species (dark gray symbol) hybridized in the middle to late Pliocene with several other species (white symbols). *(ii)*, Each hybridization event introgressed mitochondria and mtDNA in the respective receiving species, resulting in a stable polymorphism for this trait observable in extant populations. *(iii)*, Individuals with the introgressed mtDNA hybridized in the mid to late Pleistocene with another species (black symbols, typically *C. ignota* in *Calligrapha*), producing genomic mosaics with nuclear genotypes from two donors and an mtDNA genome introgressed from a third, perhaps extinct species during the ancient hybridization event. *(iv)*, Different routes to tetraploid unisexuals via genome doubling (*a*) or crossing with "conspecific" hybrids (*b*), backcrossing (*c*), crossing with additional species (pale gray symbols; *d*), or crossing with allospecific hybrids (*e*). See main text for details.

cific mating and hybridization with viable and perhaps fertile F_1 are relatively common in this genus. It is possible that these explain to great extent the extensive mtDNA species polyphyly that characterizes *Calligrapha*, but when these crosses involved females carrying the introgressed mtDNA from stage (i), it triggered a different phenomenon altogether: the potential to establish a new unisexual line. Parental analysis based on allele sharing and genome additivity suggests that these females mated with males of very few species in this stage, either *C. philadelphica* or *C. rowena* in the route to *C. vicina* or *C. ignota* in all the others.

Stage iv: polyploidization and sex loss. The actual mechanism leading to unisexuality from mosaic individuals with at least two divergent nuclear haploid genomes and a divergent mtDNA (and perhaps associated factors in the mitochondria) is not known. However, it had to involve tetraploidization via genome endoreduplication or syngamy of unreduced gametes, possibly resulting from hybrid meiotic imbalance (Comai 2005). The latter implies at least some degree of fertility in hybrid females (those who would transmit their mtDNA) and the obligate participation of males from other species or divergent populations in this initial stage. It is not possible to ascertain whether this evolutionary transition occurred in one or more generations via an intermediate triploid stage (Comai 2005), but the assorted types of genomic mosaic found among unisexual *Calligrapha* illuminate alternative possibilities.

A balanced tetraploid genomic constitution as postulated for *C. vicina* could result in a single step either from genome doubling in a diploid hybrid with introgressed mtDNA (fig. 5*a*) or by syngamy of unreduced gametes from the two parental species or their diploid hybrids (the latter requiring a relaxation of Haldane's rule, the supposedly deleterious effects of hybridization on males; fig. 5*b*). The hybrid condition with dominance of genetic contribution from one putative parental—as hypothesized for *C. alnicola*, *C. suturella*, and *C. virginea* (perhaps for *C. vicina* as well)—fits a classical model of backcrossing of hybrid mosaic females with males in the maternal parental population coupled with polyploidization, perhaps through an unstable triploid stage (Comai 2005). This model additionally explains phenotypic and ecological resemblance of the unisexual species with this parental stock due to genome additive effects (fig. 5*c*). Finally, the case of *C. apicalis*, with clear genomic contributions from at

least three bisexual lineages, requires a minimum of two successive hybridization events involving hybrid mosaic females (with introgressed mtDNA) either with males of a third species (fig. 5*d*) or with other allospecific hybrid mosaic males (fig. 5*e*).

General Implications of mtDNA Monophyly and the Sequential Hybridization Model

The proposed model implies that the first ancient hybridization event that left the signature of a distinctive mtDNA characteristic of every unisexual lineage is a necessary but insufficient condition to generate unisexuality. There was a long period of bisexuality between this event and the actual process that triggered unisexuality in *Calligrapha*, possibly caused by new hybridization events, even if the first event reinforced or conditioned the evolutionary consequences of the latter (e.g., via endosymbiont-driven selective sweep). If this or a similar process occurred in other unisexual systems, we should be cautious, regardless of apparent supporting mtDNA-based phylogenetic evidence, before invoking ancient unisexuality (also suggested for *Calligrapha:* Neiman et al. 2009; Schurko et al. 2009). The timing, phylogenetic structure, and even geographic setup described for *Calligrapha* are nearly identical to those reported for *Ambystoma* unisexual salamanders. The origin of unisexuality in this system was linked to hybridization events in the Pliocene (Bogart et al. 2007). Under our new perspective, both phenomena could actually be uncoupled. In connection with this, most studies on the evolution of unisexuality assume that the mitochondrial genome tracks the maternal lineage involved in the hybrid origin of unisexual species (Simon et al. 2003; Neiman et al. 2009). Considering the temporal delay in the sequential hybridization model proposed here, this assumption does not hold true for *Calligrapha*. Moreover, in a complex system like *Calligrapha*, mtDNA does not help in recognizing the number of origins of unisexuality, since hybrid lineages that originated independently but from the same genomic pools will likely show mtDNA monophyly (unless reciprocal crosses would result in the same unisexual phenotype).

In our current model of the evolution of unisexuality, one critical question remains unanswered: Was there a bisexual delay between the hybrid introgressed mosaic and the accumulation of polyploidy and, eventually, unisexuality? Our model implies that the second generation of hybrids in the Pleistocene had to retain at least part of its sexual reproductive potential. Otherwise, the backcrosses or crosses with additional species or with related or unrelated hybrids required by the model (and best exemplified by the origin of *C. apicalis*) would not be possible. Preliminary empirical observations from nuclear ribosomal spacer diversity also indicate that the meiotic machinery may have operated for a while to allow for gene conversion in the germ line of these lineages. In any case, if such a bisexual delay occurred, it must have been for a reduced number of generations or invoking asynaptic meiosis in order to minimize genome dilution effects (Hillis et al. 1991). But then the question remains, if there is a bisexual delay without participation of unreduced gametes, what triggered the transition from this bisexual stage to polyploidy and unisexuality? The consistent independent repetition of the same process we described is a strong indication that hybridization is a necessary condition leading to unisexuality in *Calligrapha*, but we cannot conclude that it is a sufficient condition and the actual cause for a change in reproductive mode. The model requires confirmation with analyses to reveal the actual structure of unisexual genomes in *Calligrapha*. The GISH and Zoo-FISH methods could be used to investigate genomic integrity of parental genomes in unisexual hybrid lineages. Alternatively, full-genome assemblage could be used to analyze the arrangement of linkage groups and genome-wide heterozygosity analyses used to investigate the signature of delays between hybridization and clonal reproduction. However, the confirmation of causality may certainly derive from the laboratory synthesis of the unisexual phenotypes, as achieved successfully with several vertebrate systems (Schultz 1969; Hotz et al. 1985; Lutes et al. 2011; Choleva et al. 2012). This is now feasible in *Calligrapha*, too, since our study has identified all meaningful elements for such an experiment, including relevant putative parental species as well as the unexpected requirement of females carrying the old introgressed mtDNA.

Our current understanding of the evolutionary route to animal unisexuality may be biased by the limitations of the genetic markers typically used. While data for most investigated systems suggest that hybridization is required for the evolution of unisexuality, the process may be more complicated than generally assumed. For one, hybridization may be a necessary but insufficient mechanism for unisexuality, and hybridization may occur multiple times across several temporal stages. The delay between several hybridization events and the origin of unisexuality effectively uncouples the genetic signature of hybridization from the acquisition of the new reproductive mode, and so inferences about timing, number of origins, and parenthood based on traditional markers (particularly mtDNA) can be misleading. Therefore, the origins of many unisexual systems studied to date may require a more thorough investigation.

Acknowledgments

S. Kalisz, J. A. C. Uy, and two anonymous reviewers provided highly encouraging and constructive comments to

improve the manuscript, helping us to highlight and clarify the important ideas derived from our research. In particular, we are indebted to Uy's meticulous analysis of our thoughts and useful suggestions for translating them into an understandable text. D. C. Lees (University of Cambridge, Cambridge) revised the science of this study and kindly helped with substantial idiomatic improvements to our original text. D. J. Funk (Vanderbilt University, Nashville) contributed the samples from previous studies used here. We also thank C. Ernst (McGill University, Quebec) and A. Cardoso and H. I. Vizán (Institut de Biologia Evolutiva, Barcelona) for contributing samples important to our study. This work was funded by the Spanish Ministry of Science and Innovation (CGL2008–00007/BOS, with participation of the European Regional Development Fund), including the Formación de Personal Investigador researcher training studentship to T.M. Pilot tests focusing on *ITS2* sequences were funded by the EU Synthesys initiative (FP6 Programme, Integrated Infrastructure Initiative; GB-TAF-1840) at the Natural History Museum (London).

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